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Determinants for Glycophospholipid Anchoring of the *Saccharomyces cerevisiae GAS1* Protein to the Plasma Membrane

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A 125-kDa glycoprotein exposed on the surface of *Saccharomyces cerevisiae* cells belongs to a class of eucaryotic membrane proteins anchored to the lipid bilayer by covalent linkage to an inositol-containing glycophospholipid. We have cloned the gene (*GAS1*) encoding the 125-kDa protein (Gas1p) and found that the function of Gas1p is not essential for cell viability. The nucleotide sequence of *GAS1* predicts a 60-kDa polypeptide with a cleavable N-terminal signal sequence, potential sites for N- and O-linked glycosylation, and a C-terminal hydrophobic domain. Determination of the anchor attachment site revealed that the C-terminal hydrophobic domain of Gas1p is removed during anchor addition. However, this domain is essential for addition of the glycophospholipid anchor, since a truncated form of the protein failed to become attached to the membrane. Anchor addition was also abolished by a point mutation affecting the hydrophobic character of the C-terminal sequence. We conclude that glycophospholipid anchoring of Gas1p depends on the integrity of the C-terminal hydrophobic domain that is removed during anchor attachment.

A number of eucaryotic membrane proteins are anchored to the lipid bilayer by a covalently linked glycosyl phosphatidylinositol (GPI) moiety (reviewed in references 20, 26, and 46). This particular mode of membrane attachment occurs in a wide variety of eucaryotic organisms. The modified proteins fall into diverse functional groups, including hydrolytic enzymes, cell adhesion molecules, protozoan coat proteins, and numerous cell surface antigens of unknown function.

The complete structure of the GPI moiety has been determined for two forms of the variant surface glycoprotein of *Trypanosoma brucei* (25, 58) and the mammalian cell surface antigen Thy-1 (33). GPI anchors from these distantly related organisms share a common core structure, consisting of a phosphatidylinositol molecule linked to a linear tetrasaccharide composed of one nonacetylated glucosaminyl and three mannosyl residues. At its nonreducing end, the glycan is attached via a phosphodiester to ethanolamine, which is amide linked to the α-carboxyl group of the C-terminal amino acid of the mature protein.

GPI-anchored proteins are commonly synthesized with a cleavable N-terminal signal sequence and a C-terminal domain composed predominantly of hydrophobic amino acids. This particular feature seems to be important in the mechanism of anchor addition. In all cases studied so far, addition of the GPI anchor involves the removal of 17 to 31 residues from the C terminus of a larger precursor (7, 15, 22, 27, 29, 32, 35, 36, 47, 51, 53, 60, 62, 65, 68). Since processing rapidly follows protein synthesis (2, 18, 24), it is believed that the GPI moiety is preassembled and transferred en bloc to the protein in the endoplasmic reticulum.

Several lines of evidence suggest that a signal for GPI anchor attachment resides in the C-terminal domain of the proteins. However, the C-terminal sequences of GPI-anchored proteins do not exhibit any recognizable homology. Although the structural requirements for processing remain unknown, three features may be important: a C-terminal sequence of minimal length and hydrophobicity (4, 11–13, 38, 41, 66, 73), the absence of a potential cytoplasmic domain (3, 5, 30, 31, 59, 69, 71, 72) and an acceptable anchor attachment site (52).

Recently, evidence has been provided for the existence of a similar type of membrane anchor in the yeast *Saccharomyces cerevisiae* (16, 17). A 125-kDa membrane glycoprotein of unknown function is covalently linked to an inositol-containing glycophospholipid that is responsible for membrane attachment. The composition of the yeast anchor, its sensitivity to phosphatidylinositol-specific phospholipase C (PI-PLC) and nitrous acid, and its immunological cross-reactivity suggested that it was similar in structure to GPI anchors of protozoan and mammalian origin. This finding may allow further investigation of this mode of membrane attachment by using an organism particularly suitable for genetic analysis and manipulation. We have cloned the gene (*GAS1*, for glycophospholipid-anchored surface protein) encoding the 125-kDa protein (Gas1p). The nucleotide sequence of *GAS1* was determined, and Gas1p was investigated. Our results suggest that addition of the glycophospholipid anchor to Gas1p involves removal of a hydrophobic domain from the C terminus of the polypeptide. To investigate the role of this domain in anchor attachment, mutant forms of the protein were constructed, expressed in a *gas1* null mutant, and analyzed. The data show that glycophospholipid anchoring of Gas1p depends on the presence and hydrophobic character of the C-terminal domain.

MATERIALS AND METHODS

Microbiological methods, recombinant DNA techniques, and nucleotide sequence analysis. *S. cerevisiae* strains were grown on rich (YPD) medium containing 1% yeast extract, 2% peptone, and 2% glucose. Standard procedures were followed for mating, sporulation, tetrad analysis (61), and yeast transformation (34). Transformants were selected and propagated on synthetic (SD) media prepared as described elsewhere (20a).

Plasmids were propagated in *Escherichia coli* SE10 (from S. Emr, Pasadena, Calif.) or the *dam* strain B237 (from S.

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Kvist, Stockholm, Sweden); phage M13-derived vectors were propagated in strain UT580 (from C. Lark, Salt Lake City, Utah). Standard procedures were followed for DNA manipulations (48). DNA fragments were isolated from agarose gels by using GeneClean (Bio 101, San Diego, Calif.).

DNA was sequenced by the dideoxy-chain termination method (57) with [^{35}S]dATP, using a Sequenase kit (United States Biochemical Corp.). The nucleotide sequence of *GAS1* and the deduced amino acid sequence of Gas1p were analyzed by using the programs of the University of Wisconsin Genetics Computer Group.

Isolation of the *GAS1* gene. A λgt11 yeast cDNA expression library (1) was screened with an immune serum raised against Gas1p (23a) as described previously (63). Immunoreactive clones were detected by using [^{125}I]protein A, and the phage DNA was isolated with LambdaSorb (Promega). The partial cDNA was nick translated and used as a probe to isolate the full-length gene from a yeast genomic DNA library constructed in pFL1 (14) as described previously (48). Plasmids were isolated from positive clones and introduced into strain RH100-4 (**a** *ura3 his3 suc2Δ9 pep4-3 bar1-1*). Overexpression of Gas1p was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting. The 3.4-kb *Nru*I-*Pvu*II fragment was excised from the insert of pFL1G and blunt-end ligated into the *Sma*I site of the multicopy shuttle vector pSEY8 (23) to generate pCNYG1. The same fragment was subcloned into the *Sma*I site of the *CEN* vector pSEYC58 (23) to generate pCNYCG.

Protease treatment of intact cells. Strain RH449 (α *ura3 his4 leu2 lys2 bar1-1*) was grown in YPD medium to a density of 10^6 to 10^7 cells per ml (0.1 to 1 unit of optical density at 600 nm [OD_{600}]). Cells were harvested, washed with buffer (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA, 20 mM NaN_3, 20 mM NaF), resuspended to a density of 1 OD_{600}/ml in buffer containing 140 mM cysteamine chloride, and incubated at 30°C for 20 min. The cells were then washed with buffer, resuspended to a density of 2 OD_{600}/ml in buffer containing 600 mM sorbitol, and divided into 1-ml aliquots. Proteinase K was added as indicated, and the samples were incubated at 30°C for 30 min. The reactions were stopped by addition of phenylmethylsulfonyl fluoride to a final concentration of 2 mM; total protein extracts were prepared and analyzed by SDS-PAGE and Western blotting.

N-terminal sequence determination. Gas1p was purified from strains X2180-1A (**a** *mal gal2 SUC2 CUP1*) and RH82-2 (**a** *pep4-3 bar1-1*). Cells were grown in SD medium, broken with glass beads, and extracted by using the Triton X-114 (TX-114) phase separation system (8) as described previously (17). The protein was purified from the detergent phase by affinity chromatography on concanavalin A-Sepharose (Pharmacia), followed by anion-exchange chromatography on a Mono Q (Pharmacia) fast protein liquid chromatography column (23a). At this stage, Gas1p was >90% pure, as judged from Coomassie blue- and silver nitrate-stained SDS-PAGE gels. N-terminal sequence analysis was kindly performed by J. Tschopp (Lausanne, Switzerland) by automated Edman degradation (Applied Biosystems 470A gas-phase sequenator) according to the instructions of the manufacturer. The purified protein was sequenced either directly or after preparative SDS-PAGE and electrophoretic transfer onto Immobilon sheets (Millipore) as described elsewhere (49). The sequence determined from several runs was X′-X″-V-P-A-I-E-V-V-G-N-K-F-F-Y-S-N, where X indicates an ambiguous result. Assuming that X′ and X″ correspond to D-1 and D-2, respectively, this sequence corresponds to amino acids 1 to 17 of the Gas1p sequence shown in Fig. 3.

Isolation and sequencing of [^{3}H]inositol-labeled peptides. Strain RH100-4 was transformed with plasmid pCNYG1 (see above) and grown in uracil-free SD medium to overexpress Gas1p. Under these conditions, the protein was efficiently anchored to the membrane in a phosphatidylinositol-specific phospholipase C (PI-PLC)-sensitive form (data not shown).

To label the glycophospholipid portion of the protein, cells were resuspended in inositol-free SD medium to a density of 5×10^7/ml, and 0.8 mCi of *myo*-[2-^{3}H]inositol (20 Ci/mmol; Du Pont NEN) was added. After a 1.5-h incubation at 30°C, the cells were harvested, resuspended in 10 mM Tris hydrochloride (pH 7.5)–10 mM EDTA–150 mM NaCl–10 μg each of leupeptin, antipain, and pepstatin per ml, and broken with glass beads, and a detergent phase was prepared by using the TX-114 phase separation system as described previously (17); ~90% of the incorporated radioactivity was recovered in the detergent phase, and the most prominent of the labeled proteins was Gas1p, as judged by SDS-PAGE and fluorography (data not shown).

To purify Gas1p on a large scale, cells were grown in 20 liters of uracil-free SD medium to a density of 2×10^7/ml. The cells (108 g [wet weight]) were harvested, resuspended in buffer (20 mM bis-Tris (pH 6.4), 10 mM EDTA, 10 μg each of leupeptin, antipain, and pepstatin per ml), and broken with glass beads in a Dyno-Mill. The beads were rinsed with buffer, and the homogenate (1 liter) was extracted by using the TX-114 phase separation system. The detergent phase was diluted threefold with ice-cold buffer, the [^{3}H]inositol-labeled extract (see above) was added, and the solution was applied to a DE-52 (Whatman) column (5 by 25 cm), which was washed with buffer containing 0.2% TX-114 at 4°C. Bound proteins were eluted in this buffer with 0.5 liter of a linear NaCl gradient up to 0.5 M. [^{3}H]inositol was monitored by scintillation counting, and Gas1p was identified by SDS-PAGE and Western blotting. The radioactivity and Gas1p cofractionated in the elution profile (data not shown). Peak fractions were pooled, phases were separated, and the detergent phase was analyzed by SDS-PAGE. Densitometric scanning of the Coomassie blue-stained gel showed a recovery of 4.6 mg (1.3×10^5 cpm) of ~90% pure Gas1p. An aliquot (~0.7 mg) was adjusted to 20 mM Tris hydrochloride (pH 7.5)–5 mM EDTA, and the protein was released into the aqueous phase by treatment with 22 U of *Bacillus cereus* phospholipase C (Boehringer Mannheim) for 30 min at 30°C. After another phase separation, Gas1p was precipitated from the aqueous phase with 9 volumes of ethanol overnight.

A 230-μg sample of protein was dissolved in 0.2 ml of 8 M urea–0.4 M NH_4HCO_3, reduced with dithiothreitol, and alkylated with iodoacetamide. The sample was diluted to 800 μl with H_2O and digested with 12 μg of trypsin (Boehringer Mannheim) for 4 h at 37°C; then another 12 μg of trypsin was added, and incubation was continued for 20 h. The digestion was loaded onto a C8 Aquapore RP-300 (Brownlee Labs) column (4.6 mm by 25 cm) that was then washed with 0.1% trifluoroacetic acid; ~70% of the radioactivity applied to the column was recovered in the initial wash fractions. These fractions were dried, and the pellets were dissolved in 50 mM Tris hydrochloride (pH 8), pooled, and applied to a Mono Q HR5/5 (Pharmacia) column. The column was washed with 10 mM NH_4HCO_3, and peptides were eluted with a linear gradient up to 0.5 M NH_4HCO_3 (30 min; flow rate, 1 ml/min). The radioactivity in each fraction was determined by scintillation counting. Fractions to be analyzed by sequencing were dried; the pellets were dissolved in

0.5 ml of 0.1% trifluoroacetic acid and lyophilized overnight. The pellets were washed twice with 100 μl of 50% acetic acid, dried again, and subjected to N-terminal sequence analysis by automated Edman degradation (Applied Biosystems 477A pulse liquid-phase sequenator) according to the instructions of the manufacturer.

Disruption of the *GAS1* gene. The 3.4-kb *Nru*I-*Pvu*II fragment spanning the *GAS1* gene was subcloned into pBR322 by replacing the *Nru*I-*Eco*RV fragment. The 1-kb *Xba*I-*Kpn*I fragment was then excised from the *GAS1* open reading frame(ORF), the ends of the vector were blunt-end ligated to an 8-mer *Sal*I linker, and the *LEU2* gene carried on a 2.2-kb *Sal*I-*Xho*I fragment was inserted into the *Sal*I site. This construct was cleaved with *Xho*I and *Hin*dIII, and the linear DNA was introduced into the genome of the diploid strain RH444 (**a**/α *his4/his4 ura3/ura3 leu2/leu2 tyr1/tyr1 bar1-1/bar1-1*) by a one-step gene replacement (56). Transformants were sporulated and dissected, yeast genomic DNA was prepared as described previously (55), and integration of the disrupted Δ*gas1*::*LEU2* allele at the *GAS1* locus was confirmed by Southern blot analysis (64).

Oligonucleotide-directed mutagenesis. To facilitate transfer of suitable DNA fragments between pCNYCG and M13mp18 vectors, the *Hin*dIII site immediately downstream of the *GAS1* ORF and the *Sal*I site in the polylinker of pCNYCG were removed by restriction digestion and blunt-end ligation to generate a modified pCNYCG, containing a single *Sal*I site at nucleotide 1612 of *GAS1* and single *Hin*dIII site in the polylinker. The *Sal*I-*Hin*dIII fragment, spanning the *GAS1* sequence encoding the last 159 amino acids of Gas1p and ~0.3 kb of 3′ untranslated sequence, was then subcloned into M13mp18. Two *Bgl*II sites and one *Bam*HI site were separately introduced at nucleotides 1975, 2053, and 1996, respectively (see Fig. 3) with synthetic oligonucleotides (5′-CTTGCTAGAAGATCTAGATGAAGC-3′, 5′-GC GGCAATAGATCTGGAAATGA-3′, and 5′-CGTTGGTGGA TCCATTCTTC-3′, respectively) as described previously (74), using the repair-deficient *E. coli* strain KL398 (37). DNA sequencing confirmed the desired mutations and revealed no additional changes in the *GAS1* coding region. The mutant *Sal*I-*Hin*dIII fragments were reintroduced into pCNYCG, and the C-terminal deletion was then constructed by using the vector containing the new *Bam*HI site at nucleotide 1996. After cleavage with *Bam*HI, the ends of the resulting vector were blunt-end ligated to an 8-mer *Xba*I linker with a termination codon in the appropriate reading frame, and the construction was confirmed by DNA sequencing. To express the mutant proteins, the modified pCNYCG plasmids were introduced into strain RH273-1A (Δ*gas1*::*LEU2*), derived from the disruption and dissection of the diploid strain RH444 (see above).

Protein extractions and phospholipase C treatments. Yeast strains were grown to a density of 10^6 to 10^7 cells per ml (0.1 to 1 OD_{600}). Total protein extracts were prepared as described previously (55), and the proteins were dissolved in 2× sample buffer for SDS-PAGE (40). To monitor proteins secreted into the medium, cells were resuspended to a density of 1 to 2 OD_{600}/ml in fresh medium containing 0.125 mg each of bovine serum albumin and ovalbumin per ml. After a 1-h incubation, the cells were pelleted, and proteins were precipitated from the supernatant with 10% trichloroacetic acid, washed with cold acetone, dried, and dissolved in 2× sample buffer for SDS-PAGE. To fractionate proteins by using the TX-114 phase separation system (8), cells were washed with 100 mM Tris hydrochloride (pH 7.5) and resuspended in buffer (100 mM Tris hydrochloride [pH 7.5], 2 mM EDTA, 30 μg each of leupeptin, antipain, pepstatin, and chymostatin per ml) to a density of 1 to 10 OD_{600}/ml. The cells were broken with glass beads and extracted in the presence of TX-114, and phases were separated as described previously (17). The detergent phase was diluted sixfold with cold buffer, and aliquots (from ~0.5 OD_{600} units of cells) were incubated for 30 min at 30°C with 40 U of *Bacillus cereus* phospholipase C (Boehringer Mannheim) per ml. After another phase separation, proteins were precipitated from the aqueous and detergent phases with trichloroacetic acid, and the samples were processed for SDS-PAGE.

Western blotting and affinity purification of antibodies. Proteins were separated by SDS-PAGE on 7.5% gels (40) and transferred to nitrocellulose as described previously (67). After saturation with TBS/milk (50 mM Tris hydrochloride [pH 8], 150 mM NaCl, 3% low-fat dry milk, 0.02% NaN_3), the filters were incubated for 2 to 4 h with the appropriate antibodies, washed with TBS/milk, incubated with [^{125}I]protein A for 1 h, washed again, dried, and exposed. Autoradiographs were quantified by using a computing densitometer (Molecular Dynamics, Sunnyvale, Calif.). The hexokinase antiserum was a kind gift of G. Schatz (Basel, Switzerland).

To affinity purify antibodies on the fusion protein produced by *E. coli* lysogenized with λgt11cG, the band was excised from a Ponceau S-stained nitrocellulose filter. The piece of filter was saturated, incubated with immune serum in TBS/milk, and washed as described above. Bound antibodies were eluted by washing the filter twice for 2 min each time with 0.2 M glycine (pH 2.6) and twice for 2 min each time with 0.2 M glycine (pH 2.3). The eluates were pooled and neutralized by dilution into TBS/milk.

Nucleotide sequence accession number. The sequence data reported here are available from the EMBL Data Library under accession number X53424.

RESULTS

Gas1p is exposed on the cell surface. With a few exceptions, all known GPI-anchored proteins are attached to the external face of the plasma membrane. To ascertain whether this also applies to Gas1p, we determined whether the protein was susceptible to protease treatment of intact yeast cells. Cells were treated with metabolic poisons (NaN_3 and NaF) to block vesicular traffic, and cysteamine chloride, a reducing agent that removes cell wall proteins (54), to facilitate access of exogenous protease to the plasma membrane. Proteins exposed on the cell surface were then digested by incubating the cells with different amounts of proteinase K. Total protein extracts were analyzed by SDS-PAGE and Western blotting (Fig. 1A). As a control for cell integrity, we monitored the fate of hexokinase, a cytoplasmic enzyme known to be sensitive to protease digestion. Quantitation showed no reduction in the level of hexokinase, proving that the cells remained intact and impermeable to proteinase K during the treatment. In contrast, Gas1p progressively disappeared with increasing proteinase K concentration (Fig. 1B), indicating that the protein is indeed exposed on the external face of the plasma membrane.

Cloning the gene (*GAS1*) encoding the 125-kDa glycoprotein (Gas1p). As a first step toward cloning the gene encoding the 125-kDa protein, an immune serum generated against the purified protein (23a) was used to screen a λgt11 yeast cDNA expression library. A clone containing a 1.1-kb insert (λgt11cG; Fig. 2A) was isolated and further characterized. Analysis of induced lysogens revealed that the recombinant

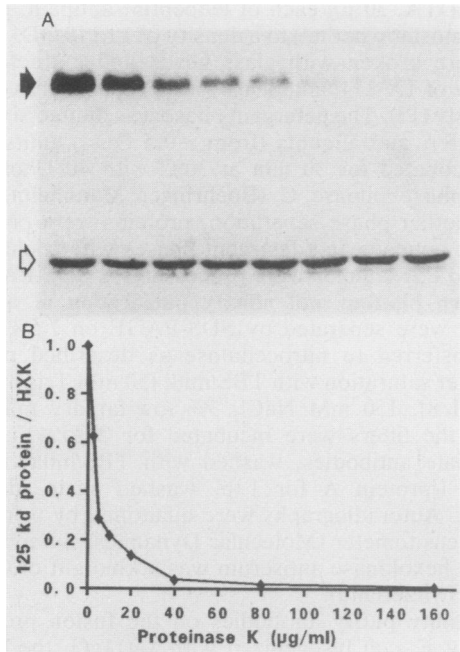

FIG. 1. Proteinase K treatment of intact cells. Strain RH449 was pretreated as described in Materials and Methods and incubated with proteinase K (0, 2.5, 5, 10, 20, 40, 80, or 160 μg/ml). Total protein extracts (from 0.5 OD_{600} units of cells per lane) were analyzed by Western blotting (A), and the autoradiograph was quantified by densitometry. The ratio of Gas1p (closed arrow) over hexokinase (HXK; open arrow) was determined for each proteinase K concentration (B).

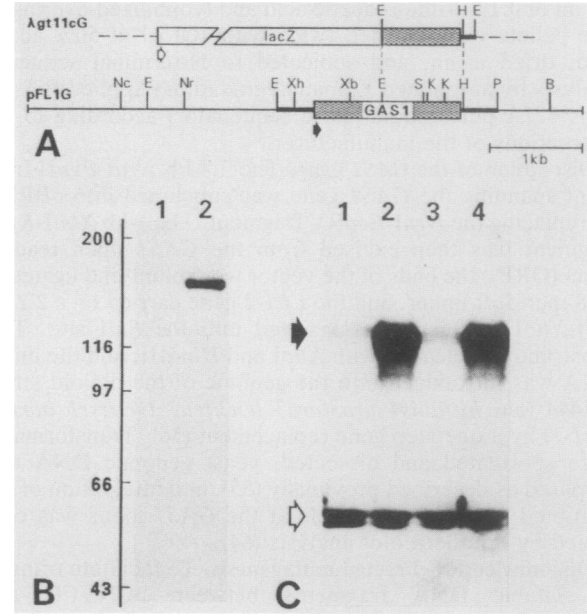

FIG. 2. Cloning of the *GAS1* gene. (A) Structure of the immunoreactive λgt11 clone (top) and restriction analysis of the genomic DNA insert of pFL1G (bottom). Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Nc, *Nco*I; Nr, *Nru*I; P, *Pvu*II; S, *Sal*I; Xb, *Xba*I; Xh, *Xho*I. (B) Identification of the β-galactosidase fusion protein encoded by λgt11cG. Total protein extracts (~50 μg per lane) prepared from induced *E. coli* strains lysogenized with λgt11 (no insert; lane 1) or λgt11cG (lane 2) were analyzed by Western blotting, using the immune serum directed against Gas1p. (C) Overexpression of Gas1p. Total protein extracts (from 0.5 OD_{600} units of cells per lane) prepared from strain RH100-4 transformed with the multicopy plasmids pFL1 (no insert; lane 1) and pFL1G (lane 2), pSEY8 (no insert; lane 3), and pCNYG1 (pSEY8 with the 3.4-kb *Nru*I-*Pvu*II fragment of the pFL1G insert; lane 4) were analyzed by Western blotting. Closed arrow, Gas1p identified by using antibodies affinity-purified on the β-galactosidase fusion protein encoded by λgt11cG; open arrow, hexokinase. Positions of molecular mass markers (in kilodaltons) are indicated on the left.

phage produced an immunoreactive β-galactosidase fusion protein of ~150 kDa (Fig. 2B). Antibodies eluted from the fusion protein specifically recognized a 125-kDa antigen in yeast total protein extracts, providing immunological evidence for the identity of the cloned cDNA (Fig. 2C). The partial cDNA was then used as a probe to screen a yeast genomic DNA library constructed in the multicopy vector pFL1. A clone containing a ~6-kb insert (pFL1G; Fig. 2A) was isolated and further characterized. When introduced into yeast cells, pFL1G directed a marked overexpression of Gas1p (Fig. 2C), indicating that this plasmid contained the full-length gene. Subcloning then revealed that the 3.4-kb *Nru*I-*Pvu*II fragment was sufficient for overexpression of Gas1p (Fig. 2A and C). No phenotype associated with overexpression of the protein was detected.

Gas1p is synthesized with a cleavable N-terminal signal sequence and a C-terminal hydrophobic domain. The 3.4-kb *Nru*I-*Pvu*II fragment (see above) contained a 1,677-bp ORF sufficient to encode a polypeptide of 559 amino acids with a molecular mass of 59.6 kDa (Fig. 3). The identity of the cloned gene was confirmed by N-terminal sequence analysis of purified 125-kDa protein. Since the first 22 amino acids of the polypeptide predicted by the *GAS1* sequence are absent from the mature protein, we conclude that this hydrophobic domain represents a cleavable N-terminal signal sequence.

In addition, the primary sequence of Gas1p contains a second stretch of hydrophobic residues comprising the last 25 amino acids of the polypeptide. Aside from the signal sequence and the C-terminal hydrophobic domain, the hydropathy profile of Gas1p (39) did not reveal any regions of significant hydrophobicity.

The deduced sequence of Gas1p contains 10 potential sites for N-linked glycosylation. Furthermore, the sequence contains, next to the C-terminal hydrophobic domain, a region rich in serine residues, which are potential sites for O-linked glycosylation. A comparison of the Gas1p sequence with known protein sequences from the NBRF protein sequence data base failed to detect any significant homology.

The glycophospholipid anchor is most likely attached to N-506 of Gas1p. In all cases described so far, GPI anchor addition occurs to the α-carboxyl group of an amino acid that is exposed after removal of a hydrophobic domain from the C terminus of the polypeptide. To provide a tracer for purification of Gas1p peptides bearing the glycophospholipid moiety, cells were labeled with [^{3}H]inositol and the protein was purified from cells overexpressing Gas1p. The purified protein was treated with PI-PLC, reisolated from the aqueous phase, and analyzed by SDS-PAGE. As determined by densitometric scanning of the Coomassie blue-stained gel, the protein was ~90% pure (Fig. 4A). Part of this preparation (~4.4 nmol; ~2,200 cpm/nmol) was digested with trypsin, and [^{3}H]inositol-labeled peptides were purified by high-performance liquid chromatography on a C8 Aquapore RP-300 column and then on a Mono Q column. Only ~60%

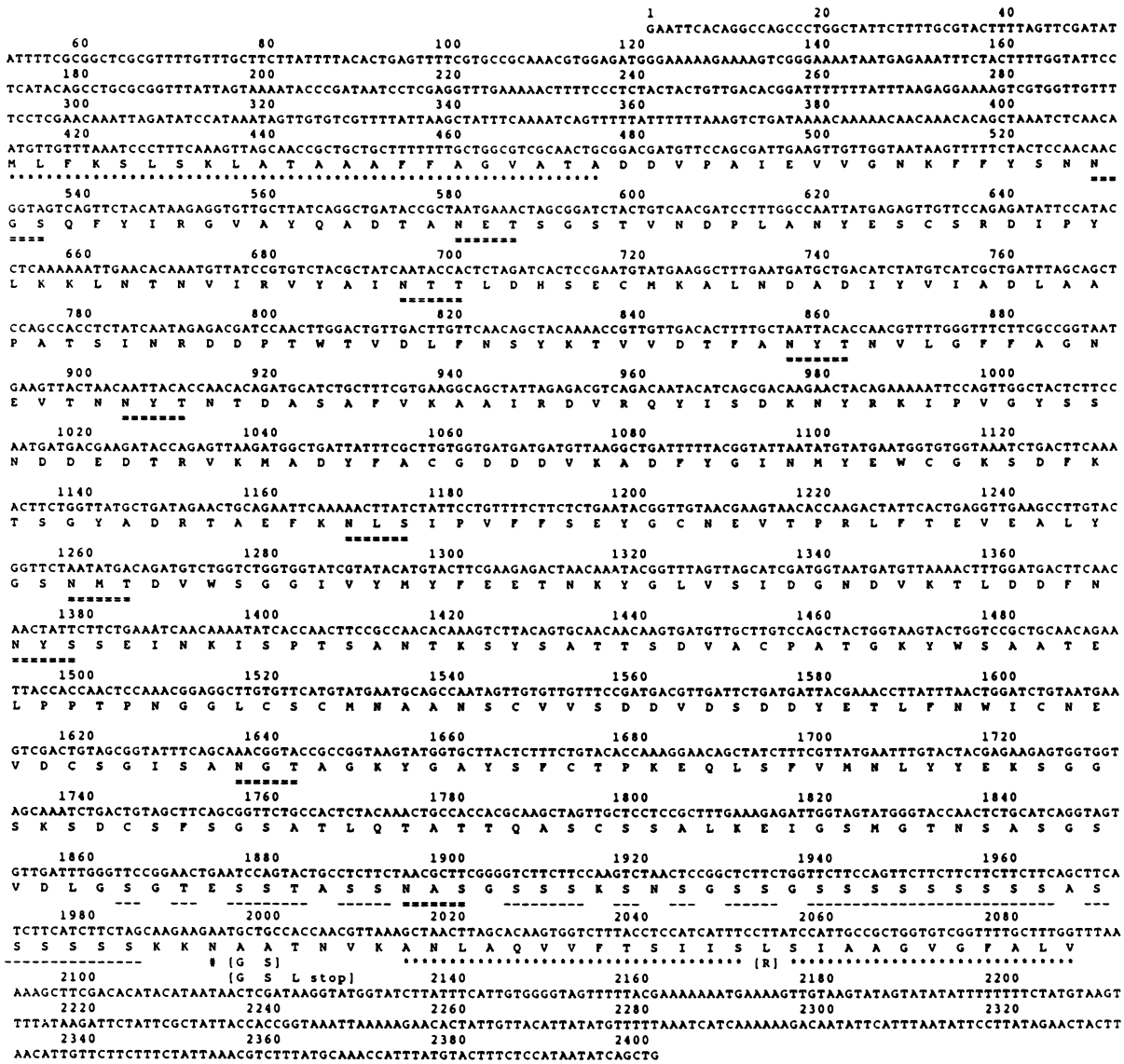

FIG. 3. Nucleotide sequence of *GAS1* and deduced amino acid sequence of Gas1p. The DNA sequence of the 2.4-kb *Eco*RI-*Pvu*II portion of the pFL1G insert (heavy line in Fig. 2A) was determined. The cleavable N-terminal signal sequence (amino acids −22 to −1) and the C-terminal hydrophobic sequence (amino acids 513 to 537) are marked (***). Potential sites of N-linked (===) and O-linked (---) glycosylation are indicated. #, Proposed anchor attachment site. The mutations that were constructed and analyzed are indicated in brackets below the wild-type sequence.

of the radioactivity bound to the anion-exchange column. This material eluted in a complex pattern between fractions 11 and 18, with peaks in fractions 14 and 17 (Fig. 4B). Fractions 14 and 17 were analyzed by N-terminal sequencing; the results are summarized in Table 1.

Fraction 14 yielded two amino acids in the first and second cycles and a single amino acid in the third and fourth cycles. This pattern is expected if the fraction contains a mixture of a dipeptide and a tetrapeptide. The tetrapeptide had to terminate in the sequence FK. Gas1p contains three FK sequences, but only one (FK-216/217) is predicted to be part of a tryptic tetrapeptide, SDFK (see Fig. 3). This is consistent with the sequencing data, and therefore we deduced that the sequence of the dipeptide was KN.

Fraction 17 yielded two amino acids in the first cycle and a single amino acid in the second and third cycles, suggesting that it was composed of a tripeptide and an additional single amino acid. The tripeptide had to terminate in the sequence EK. This sequence occurs only once in Gas1p at position 414/415 (see Fig. 3) and it is preceded by a Y, which is consistent with the sequencing information. This YEK is not predicted to be a tryptic peptide, but the N terminus of this fragment could have been generated by contamination of the trypsin with chymotryptic activity. It followed that a single N residue was the other component of this fraction.

The tetrapeptide SDFK (fraction 14) and the tripeptide YEK (fraction 17) cannot contain the anchor attachment site since each of these peptides occurred in only one of the fractions. The attachment site, bearing the [^{3}H]inositol-labeled carbohydrate portion of the anchor, has to be present in both fractions. In fact, N is the C terminus of the dipeptide

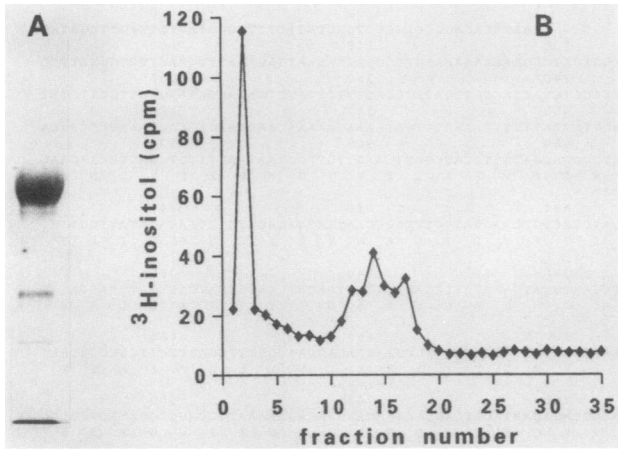

FIG. 4. Purification of Gas1p and fractionation of [^{3}H]inositol-labeled tryptic peptides. (A) Coomassie blue-stained gel showing the Gas1p preparation that was subsequently digested with trypsin. (B) Elution profile of radiolabeled tryptic peptides from the Mono Q column. The radioactivity of 1/10 of each fraction was determined by scintillation counting. Fractions 14 and 17 were subjected to N-terminal sequence analysis.

KN detected in fraction 14, and a single N is a component of fraction 17.

Several arguments favor N-506 of Gas1p as the site of anchor attachment. (i) The sequence KN (fraction 14) occurs three times in Gas1p (167/168, 230/231, and 505/506). Only one of these (KN-505/506) is located in the C-terminal region of the protein. On the basis of results obtained with protozoan and mammalian proteins, this is where one expects to find the attachment site. (ii) Only KN-505/506 is preceded by a trypsin cleavage site (K-504). If N-506 is the anchor attachment site, it is conceivable that the fragments KN (fraction 14) and N (fraction 17) were generated by incomplete tryptic digestion at KK-504/505. (iii) The absence of a trypsin cleavage site at the C terminus of the fragments is consistent with the view that these represent the C terminus of mature Gas1p. As the C-terminal amino acid is presumably amide linked to the glycophospholipid moiety, the last residue of the sequence should be the anchor attachment site. (iv) Half of the material in fractions 14 and 17 was used for sequencing (~200 and ~155 cpm, respectively). Since the specific radioactivity of the purified protein was ~2,200 cpm/nmol, the amounts of K and N (fraction 14) and N (fraction 17) found by sequencing are close to the expected values.

TABLE 1. Sequencing of tryptic peptides

Fraction	Cycle no.	PTH-amino acid[a]	Amt (pmol)
14	1	K	145
		S	51
	2	D	131
		N	102
	3	F	85
	4	K	70
	5		
17	1	N	79
		Y	151
	2	E	139
	3	K	166
	4		

[a] PTH, Phenylthiohydantoin.

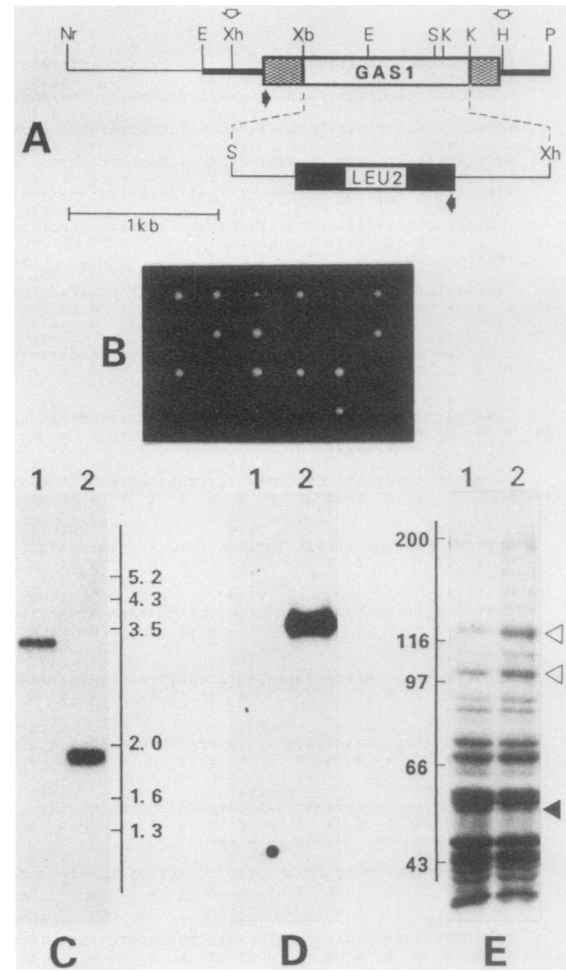

FIG. 5. Disruption of the *GAS1* gene. (A) The internal *Xba*I-*Kpn*I fragment was deleted from the *GAS1* ORF and replaced by the *LEU2* gene. The construct was cleaved at the sites indicated by the open arrows and introduced into the diploid strain RH444. Abbreviations are as in the legend to Fig. 2. (B) Tetrad analysis of RH444 heterozygous for the disrupted Δ*gas1*::*LEU2* allele on YPD medium. (C) Southern blot analysis of RH273-1A (Δ*gas1*::*LEU2*) (lane 1) and RH273-1B (*GAS1*) (lane 2). Genomic DNA digested with *Xho*I and *Hin*dIII was probed with the corresponding wild-type fragment. Positions of DNA size markers (in kilobases) are indicated on the right. (D) Western blot analysis of total protein extracts (from 0.5 OD_{600} units of cells per lane) prepared from strains RH273-1A (lane 1) and RH273-1B (lane 2). (E) Protein profiles of *gas1* null mutant and wild-type cells, showing a Coomassie blue-stained gel of total proteins (from 0.5 OD_{600} units of cells per lane) from strains RH273-1A (lane 1) and RH273-1B (lane 2). Arrowheads point to bands showing a decreased (open) or increased (closed) intensity in the *gas1* null mutant. Positions of molecular mass markers (in kilodaltons) are indicated on the left.

Disruption of the *GAS1* gene. As a first step toward elucidating the function of Gas1p, we generated a heterozygous *gas1* null mutant by replacing one of the two copies of *GAS1* in a diploid cell with a deleted and disrupted allele. A 1-kb internal fragment of the *GAS1* ORF was excised and replaced with the *LEU2* gene (Fig. 5A). Linear DNA bearing this construct was introduced into the genome of a homozy-

gous *leu2* diploid by a one-step gene replacement (56). Leu$^+$ transformants were sporulated and dissected to assess the fate of the haploid progeny.

All tetrads gave rise to four viable spores with 2:2 segregation of Leu$^+$:Leu$^-$ colonies. The Leu$^+$ (Δ*gas1*::*LEU2*) segregants gave rise to colonies significantly smaller than the Leu$^-$ (*GAS1*) segregants (Fig. 5B). Quantitation in liquid YPD medium revealed that the generation time of the mutant was increased ~20% compared with that of the wild type, and we observed that mutant cells aggregated (data not shown). Both phenotypes were reversed upon transformation of the *gas1* null mutant with a *CEN* plasmid bearing the wild-type gene (data not shown). The mutant is not defective for mating, sporulation, germination, secretion, or α-factor internalization, nor does it show any significant temperature sensitivity for growth.

Integration of the Δ*gas1*::*LEU2* construct at the *GAS1* locus was confirmed by Southern blot analysis. Leu$^-$ cells contained the wild-type gene on a 1.9-kb fragment, whereas Leu$^+$ cells contained the disrupted allele, yielding a 3.3-kb fragment of reduced intensity (Fig. 5C). Analysis of total protein extracts confirmed that Leu$^+$ segregants lacked Gas1p (Fig. 5D). Therefore, the *GAS1* gene is unique, and the function of Gas1p is not essential for cell viability.

Comparison of mutant and wild-type protein profiles indicated that lack of Gas1p affected the level of other proteins (Fig. 5E). A prominent band of ~100 kDa was less abundant in the *gas1* null mutant, whereas a band of ~55 kDa was enhanced. Preliminary results further suggest that mutant cells secrete a ~185-kDa protein that is not detected in media of wild-type cultures. Therefore, we cannot exclude the possibility that the phenotype of the *gas1* null mutant is a consequence of altered expression, stability, or targeting of other proteins.

The C-terminal hydrophobic domain of the Gas1p precursor is essential for glycophospholipid anchor attachment. To investigate the function of the C-terminal domain of Gas1p, we analyzed mutant forms of the protein generated by site-directed mutagenesis. The mutant genes were expressed in the *gas1* null mutant, and the properties of the mutant proteins were analyzed by using the TX-114 phase separation system and Western blotting.

Two alanine residues immediately downstream of the proposed anchor attachment site (N-506) were replaced by a glycine and serine residue, respectively (see Fig. 3). This protein (designated (N^{506}*GS*) complemented the growth defect of the *gas1* null mutant (data not shown). Addition of the glycophospholipid anchor was not affected, as the protein partitioned into the detergent phase and was released into the aqueous phase by PI-PLC treatment (Fig. 6A, lanes 4 to 6; Fig. 6B, lanes 5 to 8).

A protein truncated at the C terminus, containing the proposed anchor attachment site but lacking the entire C-terminal hydrophobic domain (see Fig. 3), was next analyzed. This protein (N^{506}*GSLstop*) was secreted into the medium during a 1-h incubation (Fig. 6A, lanes 7 to 9), indicating that it failed to be attached to the membrane by addition of the glycophospholipid anchor. The growth defect of the *gas1* null mutant was not complemented by the truncated gene (data not shown).

Another mutation (*R*^{526}) was constructed to assess the importance of the hydrophobic character of the C-terminal domain. A positive charge was introduced into the stretch of 25 hydrophobic amino acids that constitute the C terminus of Gas1p. The mutant gene did not complement the growth defect of the *gas1* null mutant (data not shown), and the

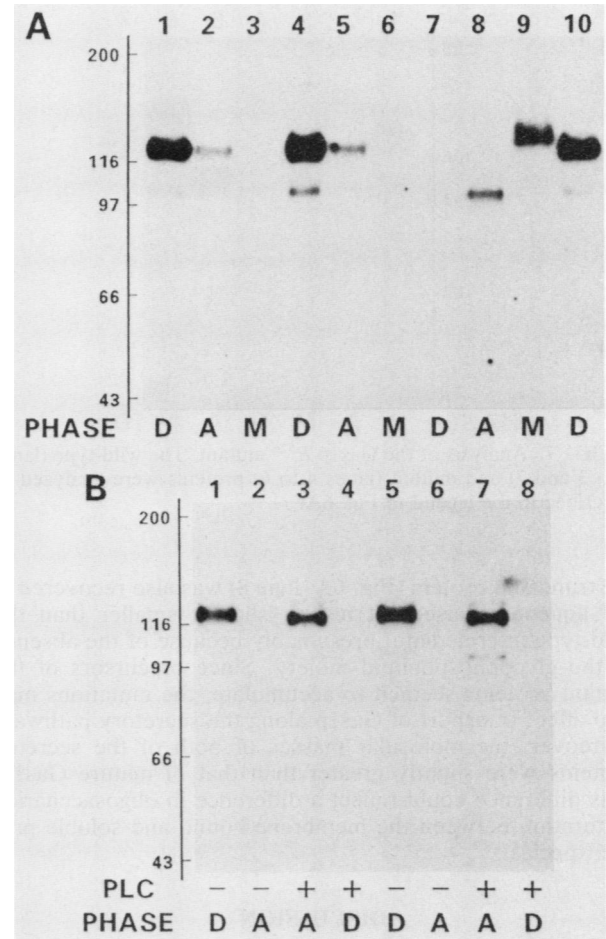

FIG. 6. Analysis of a truncated form of Gas1p. (A) Strain RH273-1A (Δ*gas1*::*LEU2*) expressing wild-type Gas1p (lanes 1 to 3 and 10), the N^{506}*GS* mutant (lanes 4 to 6), and the truncated N^{506}*GSLstop* protein (lanes 7 to 9) from a *CEN* vector (pCNYCG and derivatives) were extracted by using the TX-114 phase separation system. Proteins (from 0.5 OD_{600} units of cells per lane) recovered in the detergent (D) and aqueous (A) phases and proteins secreted into the medium during a 1-h incubation (M) were precipitated and analyzed by Western blotting. (B) PI-PLC treatment of detergent phases. The detergent phases containing wild-type Gas1p (lanes 1 to 4) and the N^{506}*GS* mutant (lanes 4 to 8) were incubated in the absence (−) or presence (+) of PI-PLC from *B. cereus*; phases were separated and analyzed as described above. Positions of molecular mass markers (in kilodaltons) are indicated on the left.

modified protein was not anchored to the membrane but secreted into the medium during a 1-h incubation (Fig. 7, lanes 4 to 6). In addition, a significant proportion of the mutant protein was recovered as a ~100-kDa species in the aqueous phase of the cell extract (Fig. 7, lane 5). A minor amount of a ~100-kDa form was also present in the detergent phase of wild-type extracts (Fig. 6A, lanes 1 and 10; Fig. 7, lanes 1 and 7). Since this species represents the core-glycosylated precursor of Gas1p (23a), it is conceivable that the intracellular portion of the mutant protein represents an analogous intermediate. However, the putative precursor of the mutant protein partitioned into the aqueous phase and was slightly larger than the wild-type species, which is consistent with the view that the protein did not undergo processing and anchor addition. The putative precursor of

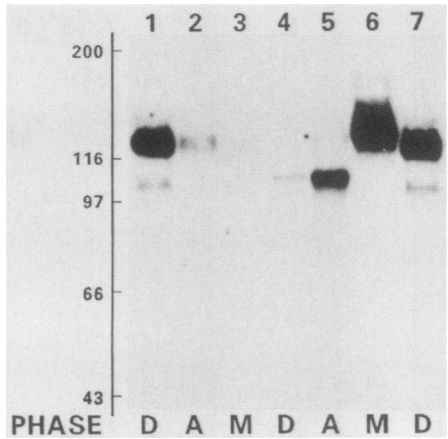

FIG. 7. Analysis of the Gas1p R^{526} mutant. The wild-type (lanes 1 to 3 and 7) and mutant (lanes 4 to 6) proteins were analyzed as described in the legend to Fig. 6A.

the truncated protein (Fig. 6A, lane 8) was also recovered in the aqueous phase, but it was slightly smaller than the wild-type intermediate, presumably because of the absence of the glycophospholipid moiety. Since precursors of the mutant proteins seemed to accumulate, the mutations may also affect transport of Gas1p along the secretory pathway. Moreover, the molecular masses of both of the secreted proteins were slightly greater than that of mature Gas1p. This difference could reflect a difference in oligosaccharide maturation between the membrane-bound and soluble protein species.

DISCUSSION

We have cloned the gene (*GAS1*) encoding the *S. cerevisiae* 125-kDa glycoprotein (Gas1p) that was previously shown to be anchored to the lipid bilayer by an inositol-containing glycophospholipid (17). Characterization of Gas1p revealed that anchor attachment depends on the integrity of a C-terminal stretch of hydrophobic amino acids that is removed from the polypeptide during anchor addition. Thus, processing of yeast Gas1p resembles that of protozoan and mammalian proteins anchored to the membrane by a GPI anchor. With a few exceptions, all GPI-anchored proteins identified so far are attached to the external face of the plasma membrane. In fact, apart from the mode of membrane attachment, this seems to be the only feature common to this functionally diverse set of proteins. The susceptibility of the protein to protease treatment of intact cells indicates that Gas1p also resides on the cell surface. It is interesting that the distribution of GPI-anchored proteins has been found to be particularly specific in polarized epithelial cells, in which the proteins are confined to the apical domain of the plasma membrane (9, 44, 45).

The primary structure of Gas1p exhibits a characteristic pattern. As shown for most of the known GPI-anchored proteins, Gas1p is initially synthesized with a cleavable N-terminal signal sequence. The signal peptidase cleavage site, which was determined by N-terminal sequence analysis of mature Gas1p, is consistent with the rules postulated by von Heijne (70). The polypeptide sequence of Gas1p can account for only ~50% of the molecular mass of the mature protein, which migrates as a 125-kDa species in SDS-PAGE. Part of this difference arises from N-linked glycosylation, since the apparent molecular mass was reduced to 95 kDa upon treatment with endoglycosidase H or F (17). In fact, the sequence of *GAS1* predicts 10 potential N-linked glycosylation sites. Therefore, it is probable that a large proportion of these sites are actually used. The remaining molecular mass difference could be due to O-linked glycosylation. In fact, the protein contains a serine-rich region next to the C-terminal hydrophobic domain (see below). Similar serine- and/or threonine-rich regions located immediately external to the membrane are known to be sites of clustered O-linked oligosaccharides in the low-density lipoprotein receptor (19), the interleukin-2 receptor (42), and the complement regulatory protein DAF (10, 50). The functional significance of this structural feature is unknown.

The serine-rich region of Gas1p is followed by a C-terminal domain composed predominantly of hydrophobic amino acids. In contrast to common membrane-spanning domains, this C-terminal hydrophobic sequence is not followed by a cluster of charged residues or a potential cytoplasmic domain. As an increasing number of genes encoding GPI-anchored proteins have been cloned and sequenced, it is now evident that these proteins are initially synthesized with a C-terminal hydrophobic domain that is absent from the mature proteins. According to the current model, the hydrophobic peptide is removed from the C terminus of the proteins and replaced with the GPI moiety during anchor addition. This was first established for different forms of the variant surface glycoprotein of *T. brucei* (7, 32), the mammalian antigen Thy-1 (60, 68), and placental alkaline phosphatase (51, 53). To date, processing of the C-terminal hydrophobic domain has been demonstrated for more than a dozen distinct GPI-anchored proteins (15, 22, 27, 29, 35, 36, 47, 62, 65).

To determine the anchor attachment site of Gas1p, C-terminal peptides were isolated and subjected to N-terminal sequence analysis. Although we were unable to purify the peptides to homogeneity, the sequencing information allowed us to deduce that the glycophospholipid anchor is most likely attached to N-506 of Gas1p. This deduction was possible only because of the presence of multiple fragments resulting from incomplete trypsin digestion. N-506 is located immediately upstream of the C-terminal hydrophobic domain. By analogy to protozoan and mammalian proteins, this is where one expects to find the anchor attachment site. Micanovic et al. (52) have recently analyzed the selectivity of the attachment site of placental alkaline phosphatase by using site-directed mutagenesis. The results showed that 6 of 16 tested amino acids (D, G, A, C, N, and S) can serve as attachment sites. These are the same amino acids found naturally as attachment sites in known GPI-anchored proteins. According to these studies, both N-506 and A-507 of Gas1p are good candidates for the site of anchor addition. However, amino acid composition analysis of the peptide fractions revealed N (detected as D) but not A (data not shown). Thus, N-506 fulfills a number of criteria for consideration as the anchor attachment site of Gas1p, and we are planning to further investigate the role of this residue by using site-directed mutagenesis.

The identification of N-506 as the anchor attachment site of Gas1p indicates that anchor addition involves the removal of a hydrophobic peptide of 31 amino acids from the C terminus of the polypeptide. This finding emphasizes the resemblance of the mechanism over a wide spectrum of organisms and supports the view that a common biosynthetic pathway has been conserved during evolution. The signals responsible for correct processing of GPI-anchored

proteins might also be conserved. To analyze the function of the C-terminal domain of Gas1p, we investigated a mutant protein that lacks the C-terminal sequence flanking the proposed attachment site. The protein did not undergo anchor attachment; instead, it was secreted into the medium. Thus, even though the C-terminal domain is ultimately removed from the polypeptide, its presence in the primary translation product is essential for anchor addition. To investigate whether the hydrophobic character of this domain is important for function, we replaced L-526 in the C-terminal hydrophobic sequence with R. Introduction of this positive charge was sufficient to abolish anchor attachment and resulted in secretion of the mutant protein. These results are consistent with data on mammalian proteins (4, 11–13, 38, 41, 66, 73) suggesting that an essential determinant for GPI anchor addition is located in the C-terminal domain of the proteins; correct function of this determinant depends on the hydrophobic character of the sequence.

At present, three other yeast proteins are candidates for glycophospholipid anchoring on the basis of their predicted protein sequences: α-agglutinin (28, 43), a cell surface protein involved in **a**/α cell agglutination during mating, the *KRE1* protein (6), involved in cell wall synthesis, and the *YAP3* protein (21), an aspartyl protease, all terminate with a hydrophobic stretch of amino acids. In the case of *YAP3*, the sequence just upstream of the hydrophobic domain contains a cluster of five serine and threonine residues followed by a KRN sequence. This is similar to Gas1p, which has five serine residues followed by a KKN sequence, where N is the proposed site of anchor attachment. The sequences of Gas1p, α-agglutinin, the *KRE1* protein, and the *YAP3* protein show no other evident homology.

This study reports the sequence and characterization of the first protein of *S. cerevisiae* that has been shown to be attached to the membrane via a glycophospholipid anchor. Our data provide additional evidence suggesting that the mechanism of GPI anchor attachment and the signal responsible for processing might be highly conserved throughout evolution. We have constructed a *gas1* null mutant and found that the function of Gas1p is not essential for cell viability. Therefore, the mutant strain can be used as a recipient to express mutant *GAS1* genes. This has permitted the analysis of mutations that abolish anchor addition and allowed us to conclude that attachment to the plasma membrane is essential for Gas1p function. Eventually, we hope that this system will enable us to use a genetic approach to identify the genes involved in glycophospholipid anchor attachment.

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